

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>5</sup> : C12Q 1/70, A61K 37/02 C07H 15/12</p>	<p>A1</p>	<p>(11) International Publication Number: WO 91/14790 (43) International Publication Date: 3 October 1991 (03.10.91)</p>
<p>(21) International Application Number: PCT/US91/02107 (22) International Filing Date: 27 March 1991 (27.03.91) (30) Priority data: 500,208 27 March 1990 (27.03.90) US (71) Applicant: NEW ENGLAND MEDICAL CENTER HOSPITALS, INC. [US/US]; 750 Washington Street, Boston, MA 02111 (US). (72) Inventor: ANDROPHY, Elliot, J. ; Two Mark Street, Natick, MA 01760 (US). (74) Agent: CLARK, Paul, T.; Fish &amp; Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).</p>		<p>(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  Published With international search report.</p>
<p>(54) Title: INHIBITORS OF TRANSCRIPTION ACTIVATION ACTIVITY OF PAPILLOMAVIRUSES</p> <p>(57) Abstract</p> <p>A method for identifying an inhibitor of the transcription activation activity of a papilloma E6 peptide. The method includes providing (a) a hybrid peptide including a papillomavirus E6 peptide having the transcription activation activity of the E6 peptide, covalently bonded with a heterologous nucleic acid binding domain having specific nucleic acid binding activity; (b) nucleic acid encoding a detectable protein in transcription relationship with nucleic acid to which the heterologous nucleic acid binding domain binds; and (c) a potential inhibitor. These three components are contacted together to cause transcription of the nucleic acid to produce a predetermined level of the detectable protein when the potential inhibitor is inactive to inhibit the transcription activation activity of that peptide; and a lower level of the detectable protein when the potential inhibitor is active to inhibit the transcription activation activity.</p>		

BEST AVAILABLE COPY

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TC	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

- 1 -

## INHIBITORS OF TRANSCRIPTION ACTIVATION ACTIVITY OF PAPILLOMAVIRUSES

### Background of the Invention

This invention relates to Papillomaviruses.

Papillomaviruses are a group of small DNA viruses that cause warts and other diseases in humans and other animals. They are implicated in the etiology of benign and malignant epithelial tumors. One group of papillomavirus is bovine papillomaviruses (BPV), another group is human papillomaviruses (HPV).

BPV type I encodes a 137 amino acid protein, termed E6, which induces morphological transformation of rodent cells in vitro; the HPV E6 protein is less efficient in causing transformation of mouse cells in vitro. The E6 proteins have moderate conservation of amino acid among various animals, and all have two pairs of cysteine repeats which are thought to mediate metal binding and the formation of zinc finger domains. These fingers are large, having 29-30 residues, and are separated by about 40 amino acids.

### Summary of the Invention

In a first aspect, the invention features a method for identifying an inhibitor of the transcription activation activity of a papillomavirus E6 peptide. These inhibitors reduce the transforming activity of the E6 peptide in vitro, and in vivo. They also block the natural life cycle of the virus, including its replication. The method includes providing: (a) a hybrid peptide including a papillomavirus E6 peptide, having the transcription activation activity of the E6 peptide, bonded, e.g., covalently, with a heterologous nucleic acid binding domain having specific nucleic acid

- 2 -

binding activity; (b) a nucleic acid encoding a detectable protein in transcription relationship with nucleic acid to which the heterologous nucleic acid binding domain binds; and (c) a potential inhibitor. These three components are then contacted together to allow transcription of the detectable protein. The hybrid peptide causes transcription of the nucleic acid to produce a predetermined level of the detectable protein when the potential inhibitor is inactive to inhibit the transcription activation activity of that peptide; and, a lower level of the detectable protein is produced when the potential inhibitor is active to inhibit the transcription activation activity.

By "transcription activation activity" is meant that the E6 peptide causes an increased level of transcription to occur when the E6 peptide is bound to the above nucleic acid by the heterologous nucleic acid binding domain, preferably in proximity to a promoter. Such heterologous nucleic acid binding domains include those binding domains which do not naturally occur in nucleic acid encoding a papillomavirus E6 peptide. Thus, one heterologous nucleic acid binding domain may be chosen from other binding domains within a papillomavirus, for example, that domain associated with the E2 peptide. This invention is not limited, however, to those binding domains found in papillomaviruses or in other viruses, since the actual binding domain chosen for use in the method of the invention is not critical. The function of this binding domain is merely to bring the E6 peptide in proximity to the nucleic acid encoding the detectable protein; that is, in a transcriptional relationship, or at a location in which the transcription activation activity of that peptide can be detected.

- 3 -

The hybrid peptide used in the method of the invention may be provided as a synthetic peptide, or provided by standard genetic engineering techniques either in vitro or in vivo. Thus, the hybrid peptide may be produced by expression of nucleic acid which encodes a papillomavirus E6 peptide (i.e., all or a portion of an E6 protein) having transcription activation activity fused to nucleic acid encoding a heterologous nucleic acid binding domain having specific nucleic acid binding activity.

The detectable protein may be any desired protein. A convenient method for detection of E6 peptide activity is to cause it to control expression of a protein that can be quantitatively measured. Thus, the protein may be an enzyme with an activity which is readily detected by in vitro analysis, e.g.,  $\beta$ -galactosidase which is detected colorimetrically. An inhibitor of the E6 transcription activation activity will reduce the appearance of color formation. Alternatively, the protein may be a lethal or an essential product, such that expression of that protein either kills a cell or allows it to survive. For example, in a preferred embodiment of the invention, the nucleic acid encoding a detectable protein is provided within a cell, the detectable protein is lethal to the cell, and the potential inhibitor is encoded by a second nucleic acid within the cell. Also provided is nucleic acid encoding and expressing a hybrid peptide. In this embodiment, only those cells which include an inhibitor of the transcription activation activity of the E6 peptide are able to grow and survive after the contacting step.

In a second aspect, the invention features a method for inhibiting the transcription activation activity of a papillomavirus E6 peptide in a patient.

The method includes administering to the patient a therapeutically effective amount of an inhibitor of the transcription activation activity of a papillomavirus E6 peptide which reduces the transcription activation activity of the E6 peptide in vitro.

In a preferred embodiment, the inhibitor which is administered is identified by the method described above.

In related aspects, the invention features a hybrid peptide including a papillomavirus E6 peptide having transcription activation activity covalently bonded with a heterologous nucleic acid binding domain having specific nucleic acid binding activity; and nucleic acid encoding a papillomavirus E6 peptide having transcription activation activity fused to nucleic acid encoding a heterologous nucleic acid binding domain having specific nucleic acid binding activity.

In preferred embodiments, the nucleic acid binding domain is chosen from a domain which binds to a promoter or enhancer region of eucaryotic DNA; and the papillomavirus E6 peptide is that E6 peptide encoded by a bovine papillomavirus or a human papillomavirus.

In yet another related aspect, the invention features an inhibitor of the transcription activation activity of a papillomavirus E6 peptide. The inhibitor reduces the transcription activation activity of the E6 peptide in vitro. Preferably, the inhibitor is an inhibitory fragment of a papillomavirus E6 peptide lacking transcription activation activity, and the peptide has a chemical moiety which covalently binds with eucaryotic DNA to prevent activation of the DNA by a papillomavirus E6 peptide; and the inhibitor interferes with the interaction of the E6 peptide with nucleic acid. For example, the inhibitory peptide comprises only one of the zinc fingers of E6.

- 5 -

This invention provides a simple way to identify inhibitors which may be used to treat warts and a variety of diseases in humans, and other animals, with compounds that are inexpensive and easy to make. The E6 peptide is present in all known papillomaviruses, thus any disease caused by a papillomavirus can be treated according to a method of the invention. Moreover, because inhibitors identified in this invention specifically block the virus to be treated, they should not adversely affect cells that are not infected with a papillomavirus.


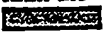

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Description of the Preferred Embodiments

The drawing will first briefly be described.

#### Drawing

The Figure is a schematic diagrammatic representation of constructions used in the examples below.

 = BPV E6 from first methionine (M) through terminal proline (P). In the E6-E2 chimeric proteins, the wild-type lysine (K) was replaced with an arginine (R) created within the oligonucleotides that fused E6 to E2. The E2 region  in E6-E2-R begins with the methionine codon at nt 3089 in BPV. E6-E2-Kif deletes 122 amino acids from the E2 protein, coupling E6 to a smaller region of the E2 C-terminus. In E6-E2-K the E2 DNA binding domain is not in-frame with E6; the terminal E6 proline is lost and replaced with 22 amino acids . (Y=tyrosine, W=tryptophan, S=serine, V=valine)

The regions of E2 required for transcriptional activation and DNA binding are bracketed at the bottom of the figure.

#### Hybrid Peptide

The hybrid peptide described above, which includes a papillomavirus E6 peptide having transcription activation activity bonded with a

- 6 -

heterologous nucleic acid binding domain having specific nucleic acid binding activity, may be produced by any standard procedure. For example, the hybrid peptide may be chemically synthesized or produced by recombinant DNA technology.

The portion of the E6 peptide having transcription activation activity can be readily identified by standard procedure. For example, various fragments of the E6 peptide may be produced by enzymatic digestion of E6 peptide, or may be synthesized in vitro by expression of fragments of nucleic acid encoding different portions of the E6 peptide. Such peptide fragments can then be fused to a heterologous nucleic acid binding domain, many of which are well known to those of ordinary skill in the art, and tested for transcription activation activity in an assay, as described below. The hybrid peptide may also be produced by chemical fusion of an E6 peptide with a heterologous nucleic acid binding domain.

Examples of heterologous nucleic acid binding domains are numerous. They include the E2 nucleic acid binding domain of papillomaviruses, and those peptides known to bind at promoter or enhancer regions. Particularly preferred are those peptides which bind to eucaryotic promoters.

Nucleic acid encoding such a hybrid peptide can be formed by standard procedure, an example of which is provided below. This example is not limiting to the present invention and those of ordinary skill in the art will recognize that many other embodiments of the invention can be provided without undue experimentation.

Example 1: BPV E6-E2 fusion peptide

This example involves construction of various hybrid peptides of the BPV E6 peptide fused to the heterologous nucleic acid binding domain of the BPV E2 peptide.



- 7 -

### E6 constructs

Referring to the Figure, BPV E6 was converted to a BamHI cassette by cloning the HpaII fragment (the restriction sites in BPV E6 are well known to those of ordinary skill in this art, see, e.g., Hawley-Nelson et al., 7 EMBO J. 525, 1988, and Vousden et al., 63 J. Virol. 2340, 1989; nucleotides [nt] 80-669) into the BamHI site of a standard vector, pUC12, after both had been made flush-ended with Klenow DNA polymerase and deoxynucleotides (dNTP's). This process formed BamHI ends and retained the first ATG-codon in the E6 reading frame. The BamHI E6 fragment and BamHI fragments from some of the constructs described below were subsequently cloned in the BamHI site of the pZipNeoSV(x)-1 vector (Cepko et al., 37 Cell 1053, 1984) or the URA-based yeast expression vector, pKP15, which included the GAL, UAS, and CYC-1 promoter, a high copy number 2 $\mu$  replication origin and the URA gene (Morrissey et al., 63 J. Virol. 4422, 1989). These expression vectors are not critical to this invention; any other suitable vector can be chosen by those of ordinary skill in this art.

### E6-E2 constructs

A BPV E6-E2-R fusion was constructed in the BamHI site of pUC12 (pUC E6-E2) by linking the BPV E6 fragment (nt 80 to nt 480, BamHI-PstI) in frame to the BPV1 E2-R fragment (Morrissey et al., supra, nt 3089 to 4450, NcoI to BamHI). This portion of E2 (called E2-R in Figure) excludes the initial 160 amino acids of the E2 transcriptional activation region. E6 was joined to E2-R through the double stranded oligonucleotide:

GA CAT GGT TCA AGG TCC AGG TAC CC

ACGT CT GTA CCA AGT TCC AGG TCC ATG GG GTAC.

This oligonucleotide forms PstI and NcoI restriction overhangs at its ends. The oligonucleotide is designed

- 8 -

to create a KpnI site by replacing the wild-type codon AAA with AGG (bold in above oligonucleotide), substituting lysine with arginine. This KpnI site is out-of-frame with a unique KpnI site in the "hinge" region of E2 (amino acid 285, nt 3455).

Digestion of E6-E2 with KpnI and re-ligation of the ends creates E6-E2-K (see figure). The terminal BPV E6 proline is lost in E6-E2-K and 22 amino acids of unrelated peptide are added to the carboxy terminus of E6.

After cleaving the KpnI site in E6-E2-K with its isoschizomer Asp718, treatment with Klenow and dNTPs, and subsequent re-ligation, the E2 DNA binding domain is restored to the E6 reading frame to form E6-E2 Kif. E6-E2 Kif excludes amino acids 161-285 of E2, which includes the residual E2 activation region present in E2-R and E6-E2-R.

An in-frame linker insertion mutation in the E2 DNA binding domain at nt 3812 (Haugen et al., 6 EMBO J. 145, 1988) was transposed into the E6-E2 chimeric vectors by substituting the NcoI (nt 3089-BstXI, nt 3888) fragment from E2-3812 (*id.*) into pUCE6-E2 to form pUCE6-E2-3812.

#### Methods

The general method of the invention is described above in the Summary of the Invention. Below there is presented an example of one such method. This example is not limiting in this invention and those of ordinary skill in the art can readily determine other embodiments of this method. In this example, the above described hybrid peptides of BPV-E6 and an E2 binding domain were used. The reporter plasmids described below are constructs having a DNA binding site (e.g., for the E2 peptide) fused to a gene encoding a readily detectable product, e.g., chloramphenicol acetyltransferase.

- 9 -

Example 2: Trans-activation in eucaryotic cells

E2-R lacks the E2 activation domain and is unable to induce E2 elements in mammalian cells and yeast (Morrissey et al., supra). The fusion protein E6-E2-R described above was constructed so that the BPV E6 peptide was essentially intact, with the only alteration of E6 limited to a conservative change of lysine to arginine two amino acids prior to its terminal proline. The C-terminal four amino acids of E6 were otherwise preserved.

NIH 3T3 or C127 cells (readily available from commercial stock centers, such as the ATCC in Maryland) were co-transfected with an E6-E2-R hybrid-encoding plasmid along with a series of chloramphenicol acetyltransferase (CAT) reporter plasmids that contain permutations of E2 binding sites. Mammalian cell reporter plasmids were provided with one, two, or three E2 binding sites (Hawley-Nelson et al., 7 EMBO J. 525, 1988), an enhancerless SV40 promoter, and the CAT gene. Transfections were carried out by calcium phosphate co-precipitation using 2-5 µg of each plasmid, and 5µg of salmon sperm DNA. CAT activity was measured by thin layer chromatography after 48 hours.

Expression of the CAT gene increased 30 to 50 fold above background levels in the presence of E6-E2-R compared to in its absence. The wild-type BPV E2 gene demonstrated activity similar to E6-E2-R with a single E2 responsive element. However, while E2 showed a significant increase in activity with two and four E2 binding sites, E6-E2-R exhibited little variation with increased numbers of binding sites.

Two distinct controls indicated that the E6 moiety of the hybrid must be localized to the promoter region for stimulation CAT expression. First, a two amino acid insertion at nucleotide 3812 in the carboxy

- 10 -

terminus of E2 inactivates the DNA binding domain of E2; this mutation is present in E6-E2-R-3812 which does not stimulate CAT production in the above protocol. Second, E6-E2-R fails to enhance transcription from a promoter with two mutated E2 binding sites as the upstream element. Further, N-terminally truncated E2 proteins, such as E2-R, do not stimulate CAT production.

Example 3: Yeast Transactivation

Cell lines were isolated that maintained both the E6-E2 and reporter plasmids through linked selectable genes. The LEU based yeast reporter plasmids pBY01, -2, and -4, that contain one, two or four E2 binding sites upstream from the CYC-1 TATA elements and the lacZ gene, were transformed in S. cerevisiae strain BGW1-7a (leu<sup>-</sup>ura<sup>-</sup>) (Morrissey et al., supra; the particular strain used is not essential to the invention). LEU<sup>+</sup> URA<sup>+</sup> colonies were initially amplified in glucose, and switched to galactose containing media 12 hours prior to assay. The levels of lacZ expression were quantified by measuring the  $\beta$ -galactosidase activity. Cells were cultured on minimal media plates with 2% galactose as carbon source and X-gal. Physiological effects of E6 were minimized since the yeast could be expanded in media supplemented with glucose, thereby repressing the GAL upstream activation sequence (UAS) that controlled papillomavirus protein expression. The ability of E2 binding site motifs to act as an E6-E2 dependent UAS when placed 5' to a lacZ gene could be rapidly and quantitatively measured with a colorimetric assay for the enzyme  $\beta$ -galactosidase.

When E6 alone (pYE6) was introduced into yeast strain BGW1-7a along with the lacZ gene that carried one, two, or four copies of the E2 binding site (pBY-1, 2, or 4 respectively; Morrissey et al., supra), no significant increase in the expression of  $\beta$ -gal over

- 11 -

levels with the vector pKP-15 was detected with cells cultured in galactose. The series of E6-E2 DNA binding domain fusion constructions were then co-transformed along with the reporter promoter plasmids and colonies that contained both constructions selected on media deficient for uracil and leucine. In comparison to basal levels,  $\beta$ -gal expression increased about 10-20 fold with E6-E2-R. By culturing the cells on galactose containing plates supplemented with the lacZ substrate, X-gal, trans-activation by E2 and E6-E2-R could be visualized. These yeast colonies were stained deep blue on indicator plates. The control constructions E2 793f (Haugen et al., 6 EMBO J. 145, 1988) and E2-R, which do not include the full E2 enhancer activation domain, failed to transactivate lacZ expression.

While one E2 binding element acted as an E6-E2-R dependent UAS, two E2 binding motifs demonstrated an additional two to three fold stimulatory effect. However, with a tandem of four sites, the expression of  $\beta$ -gal did not dramatically increase. This contrasts with BPV E2, which yielded  $\beta$ -gal quantities similar to that of E6-E2 with one or two motifs, but increased  $\beta$ -gal expression an additional five fold with four E2 binding sites.

Since the degree of activation with E6-E2-R and E2 is relatively low, a synthetic amphipathic helix (AH-E2; Giniger and Ptashne, 1987) and the strong activating region from the herpes virus VP-16 gene (VP16-E2; Sadowski et al., 1988) were cloned onto an E2 DNA binding module. VP16-E2 was constructed by amplification of the C-terminal 70 amino acids of HSV-1 VP16 (Sadowski et al., 335 Nature 563, 1988) with polymerase chain reaction (PCR) primers, and was subsequently cloned in frame onto the C-terminal 100

- 12 -

amino acid DNA binding domain of E2. AH-E2 was constructed by synthesis of complementary 50 bp oligonucleotides coding for a 25 amino acid amphipathic helix (Giniger and Ptashne, 330 Nature 670, 1987) that allowed in frame fusion at its 3' end to this E2 DNA binding region. With one or two E2 binding sites, both E6-E2-R and E2 were twice as active as AH-E2, but with four E2 motifs, AH-E2 produced a five-fold stimulation, as was observed with E2. VP16-E2 demonstrated high levels of  $\beta$ -galactosidase production that were augmented with additional copies of the E2 UAS.

Since pYE6 failed to activate the minimal promoter containing the E2 UAS, this implies that the E6 requires positioning near the transcription start site to exert its actions. As observed in rodent cells, E5-E2-R-3812 did not stimulate the E2 motifs in S. cerevisiae. E6-E2-K, in which the E2 DNA binding domain is out of translational reading frame with E6, also failed to stimulate  $\beta$ -galactosidase production. In E6-E2-Kif, 122 amino acids of the E2 enhancer activating and "hinge" domains present in E2-R were eliminated and the E2 DNA binding module was restored to the E6 reading frame. The E6-E2-Kif hybrid induced  $\beta$ -gal expression but levels were reproducibly about 40% lower than with E6-E2.

Random DNA segments from E. coli can activate transcription in yeast when localized to a promoter (Ma and Ptashne, 51 Cell 113, 1987). These activation regions are believed to form amphipathic helices with negatively charged residues on one surface. E6 does not appear to resemble these structures but instead probably exists in a zinc finger conformation.

E6 mutations (Vousden et al., supra) were transferred to the above E6-E2-R fusion gene by

- 13 -

amplification with Tag polymerase and synthetic oligonucleotides that provided a 5' BamHI and a 3' NcoI cloning site and flanked the E6 gene. Each mutation was confirmed by restriction endonuclease mapping. These E6-E2-R chimeras retain the wild-type lysine in their carboxy terminus. Three such E6 sequences E6-149, E6-368 and E60460 were transferred to the yeast E6-E2-R chimeric vector. These three mutations affect the cysteine motif at the base of each putative finger. All were transformation defective, and failed to activate the S. cerevisiae promoter. In contrast, a conservative mutation in the first finger, E6-212-E2-R was transcription competent. E6-212 has been reported to have wild-type transforming capability (Vousden et al, supra). These studies suggest that the BPV E6 transcription activation ability is concordant with its transformation properties.

The above described domain swap experiments shows that hybrid E6-E2 recognizes the E2 binding motifs ACCGNI<sub>4</sub> CCGT through the E2 DNA binding region. We also found that the E6-E2 chimera was as efficient as E6 in focus formation assays, indicating that the transcription activation properties attributed to E6 as a fusion protein do not alter its transforming potential.

The inability of the above described E6-E2 cysteine mutations to induce  $\beta$ -galactosidase production is evidence that trans-activation is dependent on an E6 domain, and that the folding of E6 into a zinc finger conformation is a prerequisite for its transcriptional function. The zinc finger motif may function as a transcriptional activator.

The methods described above, in which a DNA binding site is fused to an E6 peptide and used to effect increased transcription from various promoters in

- 14 -

various cell systems can be used to identify inhibitors of the transcription enhancing activity of the E6 peptide. This can be performed generally as described above in the Summary of the Invention. Generally, a system similar to those described in the above examples is used to test a variety of peptides, or other potential inhibitors of the E6 activity. The amount of transcription from the reporter gene is measured in the presence, and in the absence, of such potential inhibitors. Those inhibitors which reduce the amount of transcription are potentially useful for inhibition of the activity of E6 in vivo. These potential inhibitors can be chosen from any number of compounds, including segments of the E6 peptide. Such segments may be created by enzymatic digestion of the E6 peptide, purification of the peptide fragments and then use of those peptide fragments in a transcriptional assay. In addition, synthetic peptides may be synthesized and tested in the assay. Any desired peptide can also be produced by expression of nucleic acid encoding a peptide by standard recombinant DNA technology. It is also desirable to test inhibitors which are designed to chemically interact with E6 in vivo to permanently inactivate the E6 activity. Such compounds will form covalent or ionic bonds with the E6 peptide, and can be constructed by standard chemical procedures.

#### Use

The hybrid peptides and nucleic acid encoding those peptides can be used in the method of the invention for identifying inhibitors of the transcription activation activity of the papillomavirus E6 peptide. These inhibitors can then be introduced in any standard therapeutically effective amount to a patient infected with a papillomavirus. Cells infected



- 15 -

with a papillomavirus can be saturated with the inhibitor to prevent the E6 protein produced by that papillomavirus interacting with nucleic acid within the cell, and enhancing the expression of that nucleic acid. Such inhibition results in inhibition of the tumor forming activity of the virus.

The inhibitors can be provided in pharmaceutically acceptable media to be applied to regions infected by the papillomavirus, or for injection into an animal. They are especially useful for application to a human wart. The inhibitors may be either free in solution, or DNA encoding the inhibitors ligated to the DNA of a non-pathogenic virus for transfection into an infected cell. Alternatively, a small amount (e.g., about 0.1-10µg) of an inhibitor preparation (e.g., the inhibitor dissolved in DMSO and/or saline) may be allowed to penetrate viral infected cells (e.g., by the method described in 82 Proc. Nat. Acad. Sci. (USA) 2781, 1986) by applying the preparation to an infected region; preferably the preparation contains protease inhibitors, e.g., EDTA, to prevent enzyme activity.

Peptides containing a papillomavirus E6 inhibitor can also be used to inhibit the growth and expression of a papillomavirus. The peptide can be introduced into viral-infected cells so that it can bind to the E6 transcriptional activation sites and prevent native E6 protein from binding to these sites. A peptide containing such binding sites can be used to treat papillomavirus infection. The peptides can be dissolved in a pharmacologically-acceptable buffer and applied to infected cells. DMSO and EDTA can be used to help the uptake of the peptide and to inhibit protease

- 16 -

degradation. Alternatively, the peptide can be fused chemically, or by standard genetic engineering techniques, to a cell specific receptor peptide, e.g., epidermal growth factor, so that the peptide is more readily taken up by cells. Further, the peptide may also be fused to a nuclear targeting sequence (see 7 Mol. & Cell. Bio. 2451 (1987); 39 Cell 499 (1984); 46 Cell 575 (1986); 6 Mol. & Cell. Bio. 4136 (1986); 311 Nature 33 (1984) so that the E6-protein fragment is transported to the cell nucleus where it can inhibit viral growth. Preferably, the peptides are applied in the range of 1-1,000  $\mu$ g per kg animal, or at 1-1000  $\mu$ g/ml when used topically.

Thus, inhibitors of the present invention are useful for prevention of formation of cancers or growth of cancers including penile cancers, anal cancers, oral cancers, pharyngeal cancers, tongue cancers, epithelial, especially squamous epithelial cancers and some skin cancers.

Other embodiments are within the following claims.

- 17 -

Claims

1. A method for identifying an inhibitor of the transcription activation activity of a papillomavirus E6 peptide, comprising the steps of:

providing a papillomavirus E6 peptide having transcriptional activation activity bonded with a heterologous nucleic acid binding domain having specific nucleic acid binding activity,

providing nucleic acid encoding a detectable protein in transcription relationship with nucleic acid to which said heterologous nucleic acid binding domain binds,

providing a potential inhibitor,

contacting said hybrid peptide with said nucleic acid in the presence of said potential inhibitor to cause transcription of said nucleic acid to produce a predetermined level of said detectable protein when said potential inhibitor is inactive to inhibit said transcription activation activity, and to produce a lower level of said detectable protein than said predetermined level when said potential inhibitor is active to inhibit said transcription activation activity.

2. The method of claim 1, wherein said nucleic acid is provided within a cell, said detectable protein is lethal to said cell, said potential inhibitor is encoded by a second nucleic acid within said cell, and only a cell comprising said inhibitor survives after said contacting step.

- 18 -

3. A method to inhibit the transcription activation activity of papillomavirus E6 in a patient, comprising the steps of:

providing a therapeutically effective amount of an inhibitor of the transcription activation activity of a papillomavirus E6 peptide which reduces the transcription activation activity of the E6 peptide in vitro; and

administering said inhibitor to said patient.

4. A hybrid peptide comprising a papillomavirus E6 peptide having transcription activation activity covalently bonded with a heterologous nucleic acid binding domain having specific nucleic acid binding activity.

5. The hybrid peptide of claim 4 wherein said nucleic acid binding domain is chosen from a domain which binds to a promoter or enhancer region of eucaryotic DNA.

6. The hybrid peptide of claim 4 wherein said E6 peptide is that peptide encoded by BPV or HPV.

7. Nucleic acid encoding a papillomavirus E6 peptide having transcription activation activity fused to a heterologous nucleic acid binding domain having specific nucleic acid binding activity.

8. The nucleic acid of claim 7 wherein said nucleic acid binding domain is chosen from a domain which binds to a promoter or enhancer region of eucaryotic DNA.

- 19 -

9. The nucleic acid of claim 7 wherein said E6 peptide is that peptide encoded by BPV or HPV.

10. An inhibitor of the transcription activation activity of a papilloma virus E6 peptide, said inhibitor reducing said transcriptional activation activity of said E6 peptide in vitro.

11. The inhibitor of claim 10 comprising an inhibitory fragment of a papillomavirus E6 peptide lacking transcriptional activation activity.

12. The inhibitor of claim 11 wherein said peptide has a chemical moiety which covalently bonds with eucaryotic DNA and prevents activation of said DNA by a papillomavirus E6 peptide.

13. The inhibitor of claim 10 wherein said inhibitor interferes with the interaction of said E6 with nucleic acid.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 91/02107

<b>I. CLASSIFICATION</b>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): C 12 Q 1/70; A 61 K 37/02; C 07 H 15/12		
U.S. Cl.: 435/5; 530/324; 536/27		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched		
Classification System	Classification Symbols	
U.S.	435/5, 69.1, 41, 71.1, 71.2, 70.3; 530/324; 536/27	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages "	Relevant to Claim No. "
X	EP, A, 0,302,758 (ANDROPHY ET AL) 08 FEBRUARY 1989, see entire document.	1-13
<p>* Special categories of cited documents:</p> <p>"A" document defines the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority, (prior art) or which is cited to establish the publication date of an invention or other special reason (is specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the invention but "T" used to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to be an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to be an inventive step when the document is considered with other prior art other such documents, such combinations being obvious to a person skilled in the art</p> <p>"Z" document mentioned in the same prior art</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		
14 MAY 1991		
International Searcher		
ISA/US		
Date of the Actual Completion of the International Search		
25 JUN 1991		
Bradley L. Suson		
BRADLEY L. SUSSON		

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**